THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR *Françoise Beaulieu*

TITLE OF THESIS *DETERMINATION OF BHA AND BHT IN DEHYDRATED MASHED POTATOES*

DEGREE FOR WHICH THESIS WAS PRESENTED *Master of Science*

YEAR THIS DEGREE GRANTED Spring 1982

THE UNIVERSITY OF ALBERTA

DETERMINATION OF BHA AND BHT IN DEHYDRATED MASHED POTATOES

by

© *Françoise Beaulieu*

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF *Master of Science*

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Spring 1982

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *DETERMINATION OF BHA AND BHT IN DEHYDRATED MASHED POTATOES* submitted by *Françoise Beaulieu* in partial fulfilment of the requirements for the degree of *Master of Science* in *Food Chemistry*.

ABSTRACT

The extraction step in BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene) analysis of potato granules, unless precautions are taken, leads to a recovery of less than 10%. This study showed that BHA (BHT) is retained in granules by association of leached-out amylose chains during the conditioning step of the Add-Back process. X-ray diffraction data substanciated that amylose contained entrapped rather than inclusion clathrate forms of BHA. No satisfactory recovery was obtained using solvents of increasing dielectric constant unless the granules were first hydrated with water. A rapid antioxidant extraction procedure, based on the hydration principle and suitable for quality control labs, is described. Comparative data were acquired for the content of BHA (BHT) in potato granules analysed by differential pulse voltammetry (using a glassy carbon electrode) and gas-liquid chromatography. A voltammetric method of assay was also used for major potato phenolics.

RÉSUMÉ

Les méthodes actuelles d'extraction des antioxydants (BHA ou BHT) contenus dans les granules de pommes de terre s'avèrent trop souvent incomplètes puisque moins de 10% de la quantité totale présente est extraite. On a demontré dans cette étude que les antioxydants se révèlent emprisonnés suite à une association des chaînes d'amylose libres durant la phase de conditionnement du procédé de déshydratation des pommes de terre. Les résultats obtenus lors d'études radiocristallographiques ont indiqué que la nature de cet emprisonnement est attribuable au réalignement des chaînes d'amylose plutôt qu'à la formation de complexes d'inclusion.

La récupération des antioxydants, en dépit de l'utilisation de solvants à constantes diélectriques croissantes, n'a pu s'accomplir totalement que par une hydratation préalable des granules de pommes de terre.

Les analyses qualitatives et quantitatives ont été effectuées avec l'aide de la polarographie à pulsation différentielle et la chromatographie en phase gazeuse. Dans le cas de l'analyse des majeurs composés phénoliques des pommes de terre, la polarographie s'est avérée particulièrement prometteuse.

Finalement, l'application du principe d'hydratation a permis la mise au point d'une technique d'extraction qui offre des avantages certains pour les laboratoires de contrôle de qualité.

ACKNOWLEDGMENT

I wish to express my special appreciation and deepest gratitude to Dr. D. Hadziyev, my supervisor, for his counsel and encouragement throughout the course of this study. His critical review of the manuscript is also deeply appreciated.

I gratefully acknowledge the financial support offered by the Department, in the form of a Graduate Research Assistanship, which made completion of this project possible.

Special thanks are due to Dr. G. Packer, Dr. B. Ooraikul, Dr. P. Sporns and Dr. A. Horak for helpfull discussions and for their service as committee members.

I would like to express my gratitude to Mr. L. Steele and M. J. Bourgois for their untiring assistance in the proof reading and computer formatting of the manuscript.

My sincerest acknowledgement is also extended to all other members of the Department, whose encouragement and friendship meant so much to my invaluable introduction into Shakespeare's language.

Last, but certainly not least, I would like to express my indebtedness to my companion, Christian, for his minimal complaints, infinite compassion and constant support during my times of great need.

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I. INTRODUCTION

The role of phenolic antioxidants in potato granules is to prevent oxidative rancidity of the natural fat of the potato during shipment and storage. The amount allowed in foods is subject to government regulation. Guidelines for dehydrated potato granules permit, for BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene), or their mixture, not more than 50 ppm (Canada), 10 (USA), or 25 for the U.K. and France. The trend in granule production is to decrease the proportion of BHT or omit it completely. In the Add-Back process, antioxidants are usually applied with a formulated emulsifier added to cooked potatoes in the mash-mixing step. Some plants apply BHA as an ethanol solution and BHT as small crystalline beads. Steam distillation and volatilization occurring during the Add-Back process cause tremendous losses; the net retention of BHA in the granules has been estimated to amount to 40-50%, while for BHT only 2-5% of the quantity added is left in the final product. The exact nature of the retention of antioxidants in dry granules is unclear.

Analytical methods for BHA (BHT) determination in granules can be unreliable and most are long and time consuming (Bieth et al., 1978; Halot, 1971). The AOAC method (Takahashi, 1970) gives results with 40-80 % less antioxidant than methods based on continuous extraction of granules. Quality control laboratories lack a reliable, economical and, above all, a fast method which can be

applied in the plant and also be recognized as valid on the export market.

This study elucidates the status of antioxidant retained by model systems consisting of major potato constituents. Also, it describes a rapid and quantitative method for extraction of antioxidants in potato granules, followed by their determination by voltammetry using a glassy carbon electrode and by gas-liquid chromatography (GLC). In addition comparative data are provided for the content of BHA (BHT) in granules manufactured by an Add-Back granule process.

II. REVIEW OF LITERATURE

A. *Potato granule production*

With regard to nutritive value and extent of cultivation, the white potato (*Solanum tuberosum* L.) is the most important single vegetable. Therefore, preservation processes have been developed to retain the nutritive value of potatoes over long periods of time. Currently, over 50% of Alberta's crop is processed, indicating an ever increasing trend to potato consumption in varied forms such as granules, French fries and chips. For example, in 1978, close to 14% of the total production was processed into granules (Krahn,1980).

Potato granules are a dehydrated product made from freshly cooked, mashed potatoes. They have a high bulk density (0.94-0.96 g/cm^3) which reduces costs of packaging, shipping and storage. Development of a dehydrated potato granule process started in England during the latter part of World War II (Greene et al., 1948). It is always the aim to produce granules which, on mixing with hot water, rapidly form a dish of mealy texture comparable to freshly prepared mashed potatoes. However, this is difficult to do commercially; the problem being that the reconstituted mash tends to be sticky and pasty instead of mealy. This undesirable effect is caused by a rupture of cells during processing, thereby releasing starch--it is this extracellular starch (mostly amylose) which gives the

product its pasty texture. Cell rupture occurs mainly in the steps of the process where the potato material is subdivided (Gutterson, 1971). Various procedures have been advocated to attain the desired subdivision with a minimum of cell rupture. Many patents have been issued and they were adequately reviewed by Olson and Harrington (1955), Feustel et al.(1964), Gutterson (1971) and Hanson (1975). At present, the only method which has come into commercial production of potato granules is the Add-Back process.

1. The commercial Add-Back process

The process, originally patented by Volpertas (1944) and Rendle (1945), is carried out by admixing cooked mashed potato with an approximately double amount of the dried granules, thereby preparing a friable mixture that can be dried to a powder. The principle is that, since the moisture content of the cooked mashed potato is reduced to 50% or less, its adhesive characteristics are minimized to the point where conventional drying procedures can take place without substantial cell rupture,thus forming a dehydrated product consisting largely of individual potato cells or aggregates of several cells (Hanson, 1975).

The Add-Back process basically involves: steam-peeling, trimming, slicing, washing, precooking and cooling, steam-cooking, mashing and mixing with dry granules and additives, conditioning, remixing, air lift drying, fluidized bed drying, cooling and sifting (Figure 1). The

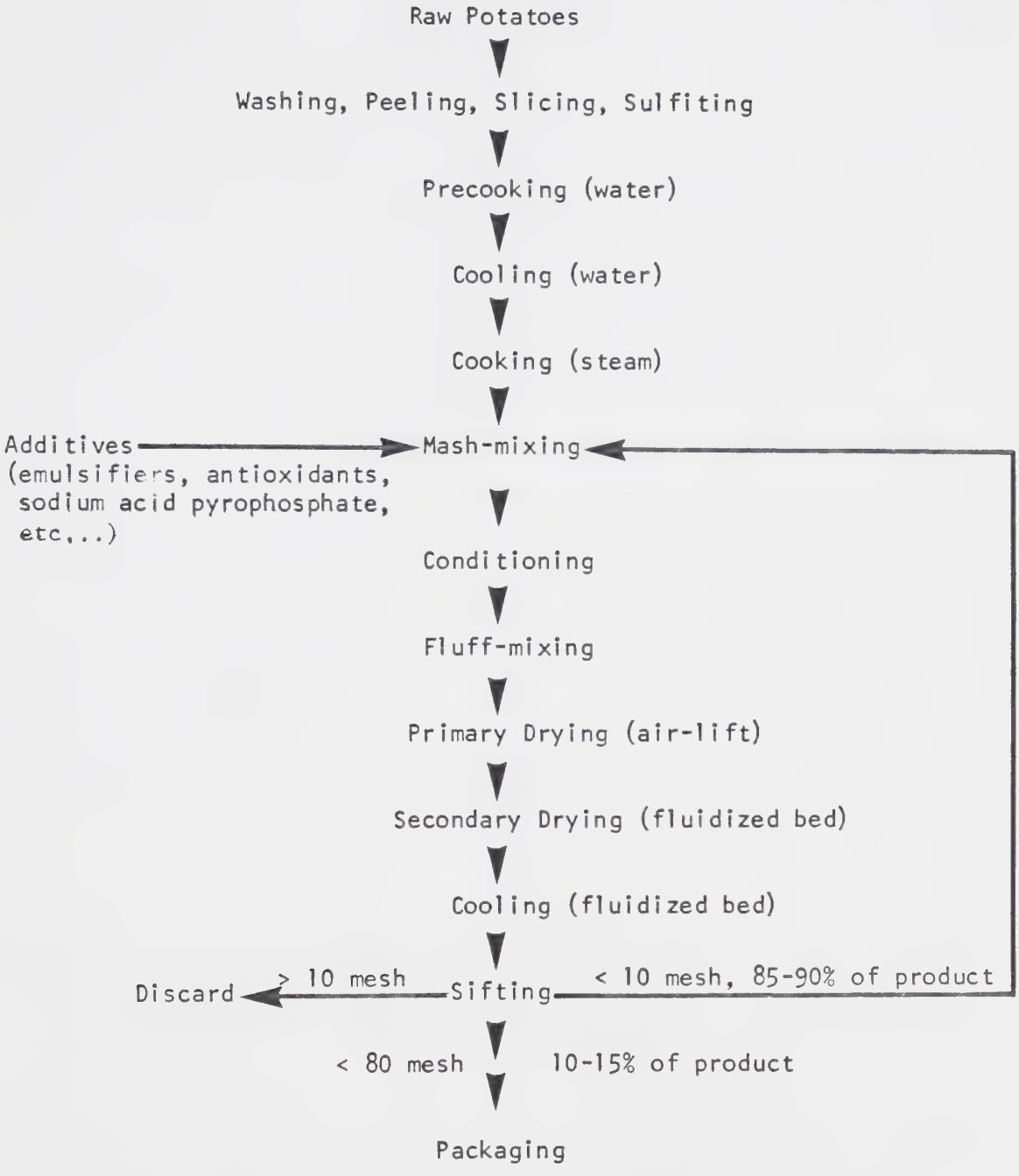


Figure 1. Flow chart of a commercial Add-Back process.

following description outlines the rationale for the sequence of steps: slicing ensures effective and uniform heat transfer during cooking. Precooking in water at 60-80°C for 20-30 min, followed by cooling for 20 min in tap water causes the gelatinization and the retrogradation of the starch (Potter et al., 1959). This serves to avoid sloughiness during cooking and to enable the potato cells to withstand the forces generated by compression, mixing and rubbing during the continuous mash-mixing step. Steam-cooking brings about final softening of the tissue. Hot mash-mixing results in tissue separation into individual cells or their aggregates, with minimum cell rupture. Additives such as sulfite, sodium acid pyrophosphate, monoglycerides and antioxidants are incorporated at this step. Conditioning in a stream of cool air for about 45 min is needed, firstly, to equilibrate the moisture between the freshly cooked and the previously-dried material. Secondly, it forces the free starch to retrograde and thus aids in the granulation of the moist mash. The object of remixing is to "fluff" the mix and to break down the loose agglomerates which have formed during the conditioning period in order to produce essentially single cell particles. Air lift drying reduces the moisture content of the granules from 30 to about 15%, while the following fluidized bed drying brings it down to about 7%. The cooled granules are then sieved. A small portion (1/10 to 1/6) of particle size 80 mesh or less is collected as end product, while the remaining, greater

part of the output, is recycled. The fraction of particles of 10 mesh or greater is discarded or used as an animal feed ingredient (Toffolo, 1968).

It has been estimated that the granules have to endure an average of 8-10 processing cycles before they are collected as final product. Accumulated physical and nutritional damage can be quite considerable (Moledina et al., 1978b). Constant recirculation of the granules obviously has a bearing on operating costs. Therefore, "straight-throught" processes have been developed and patented in order to attain subdivision of cooked tissue without the shear action of the Add-Back granules.

2. The Freeze-Thaw process

The beneficial effects of freezing and thawing steps in potato granule production have long been recognized (Rendle, 1945; Willetts and Rendle, 1948; Greene et al., 1948). Rendle (1945) suggested their introduction as an optional step prior to admixing of the dry granules.

In the freeze-thaw process, the precooking and cooling steps are not essential and can, in fact, be considered detrimental since they inflict substantial cell damage at the mashing step (Moledina, 1978a). Greene et al., (1948) found that remarkable toughening of the cell wall as well as formation of free moisture (50%) can be achieved without damaging the cells, by freezing and thawing the cooked mashed potatoes.

A direct technique for production of potato granules, using a freezing and thawing step as an integral part of the process, was patented by Ooraikul (1977). The basic features of the process are: peeling, slicing, washing, sulfiting, steam-cooking, mashing along with additives, freezing in an air-blast freezer at -29°C, thawing at room temperature immediately followed by predrying, granulation and final drying step (Figure 2).

The final product exibits a high proportion of 60 mesh particles, a very low broken cell count (3%) and, normally, less than 1% discard. The reconstituted product is organoleptically similar to freshly cooked and mashed potatoes (Ooraikul, 1977).

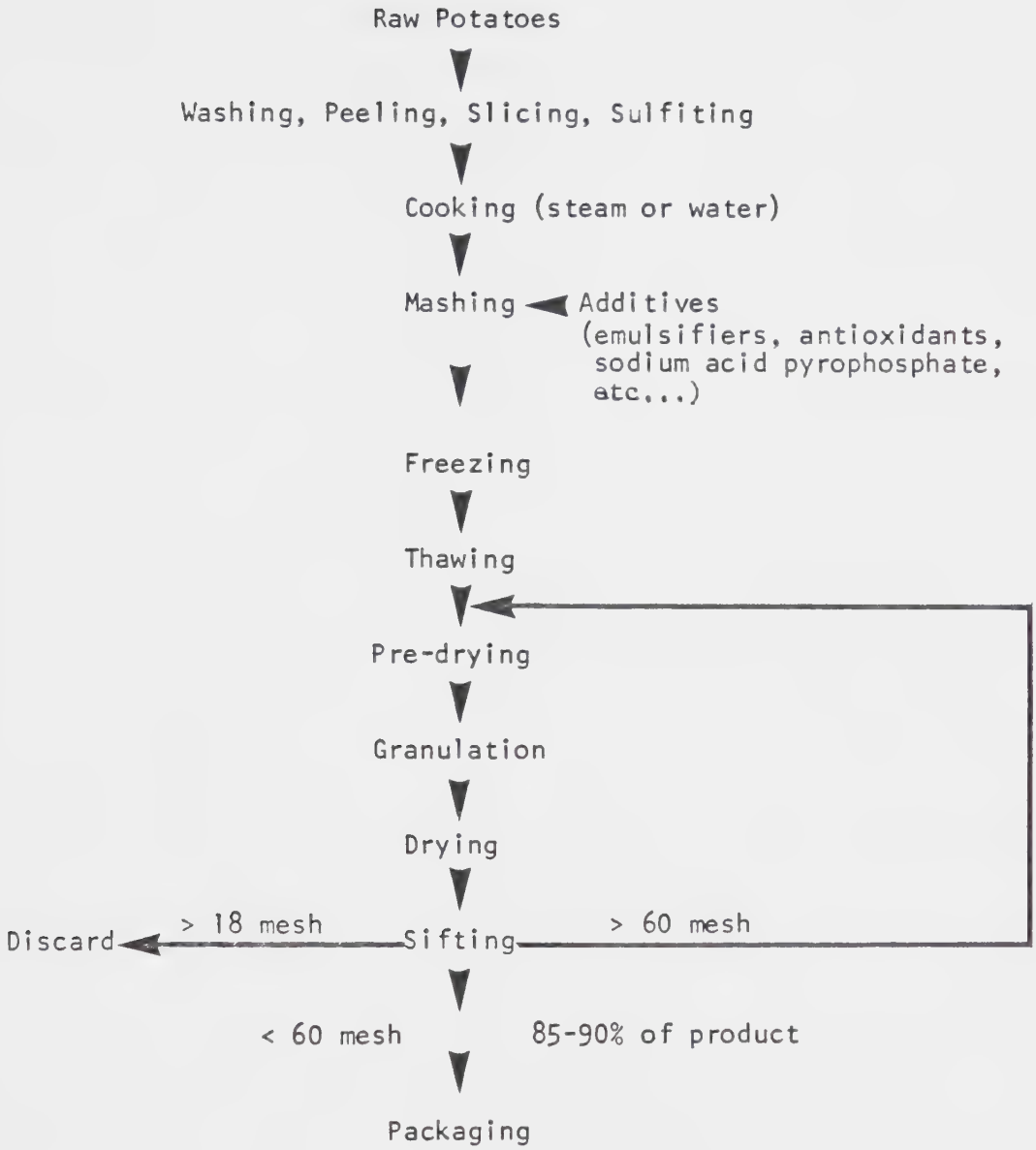


Figure 2. Flow chart of a Freeze-Thaw process.

B. *Rancidity problems*

1. Lipids in raw and granulated potatoes

Lipids are a trace constituent in potatoes. The average content of ether-extractable matter of the potato ranges from 0.02 to 0.2% on a fresh weight basis (Talburt and Smith, 1975). Gaillard (1968) indicated that the total lipid extracted with propanol and chloroform-methanol represented approximately 0.13% (fresh weight) of the tuber.

A detailed study on the identification and quantitative determination of the lipids in Canadian cultivars of potatoes was provided by Lepage (1968). The total lipid content was found to be relatively low for all cultivars (about 0.5%, dry weight). The results showed that neutral lipids in the Netted Gem variety amounted to 16.5%, whereas the phospholipids and glycolipids were 45.5 and 38.0%, respectively. The major lipids in the polar fraction were phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, the galactolipids and the sterol glucosides. Furthermore, no significant difference was found between the overall fatty acid composition of the four cultivars tested; linoleic (52.0%), linolenic (24.2%), palmitic (17.5%) and stearic (4.9%) being the predominant fatty acids.

Lepage (1968) also stressed that, since lipids in potato are mostly polar, a polar solvent must be used for total lipid extraction. Swoboda (1973) stated that, whereas solvents such as petroleum or diethyl ether adequately

extract neutral lipids (i.e. triglycerides, etc.) more powerful mixed solvents such as chloroform-methanol or water-saturated butanol are required to extract the polar complex lipids (i.e., phospho- and galactolipids, etc.). The author further indicated that this is particularly the case for cereal or vegetable products, which have a very low total lipid content, with a high proportion of complex lipids.

Talburt and Smith (1975) reported that a portion of potato lipids may be bound to other constituents. Gaillard (1973) pointed out that phospho- and glycolipids are those associated with lipoprotein membrane structures. Consequently, extraction with chloroform-methanol yielded 0.5% lipid (dry weight) in comparison with 0.2-0.26% when petroleum ether was used (Lepage, 1968). Doubts were raised about results from earlier studies where a nonpolar solvent was used in lipid extraction (Lampitt and Goldenberg, 1940; Kröner and Völksen, 1950; Highlands et al., 1954).

Transmission electron microscopy data for potato cells obtained before and after extraction with selective lipid solvents were provided by Chung et al.(1979). Potato samples extracted at room temperature with acetone, known to be a poor phospholipid solvent, showed a complete loss of amyloplast membranes, peroxisome membranes, lipids in cell walls and rare lipoid bodies distributed in the cytoplasm. Similar results were obtained with potato samples washed with petroleum ether. Extraction with chloroform-methanol

(2:1 v/v) removed all the lipid membranes, while n-butanol and methanol-ethanol (1:1 v/v) washed out all the cell membranes.

The lipids of white potatoes are a complex mixture, including phospholipids, glycolipids, sterols and sterol glucosides, glycerides and free fatty acids. Mondy and Mueller (1977) investigated the effect of cooking methods on the lipid composition of potatoes. Results showed that cooking did not alter the fatty acid composition. However, they observed that the crude lipid content of potatoes, including both pith and cortex tissues, was lowered significantly from that of the fresh tissue. They related the loss of lipids to the degree of histological changes associated with cooking. They suggested that cell wall distention and rupturing may permit lipids to diffuse from the tissue. Lipids which function as structural components of cellular organelles, e.g. phospho- and galactolipids, would not be free to escape from the tissue except when fragmented from the lipoprotein membranes as a result of cell rupturing.

Little is known about lipids in processed potatoes. Distribution, composition, nature and unsaturation ratios of lipids in the newly-acquired cell structure are the main points of interest. As stated by Hadziyev and Steele (1979), the exact spatial arrangement of lipids in dehydrated granules has not been fully established.

Recently, a detailed study of the lipid composition and constituents in raw and granulated potatoes was provided by Pun (1979). Transmission electron microscopy revealed that lipids in cooked potato cells exist in the form of membranes sandwiched between gelled starch and the cell wall. No imbedded lipid was detected within the gelled starch. Steam-cooked tissue did not show cell distention or cell rupture, but exhibited substantial change in the spatial organization of the lipids. The author suspected that this new cellular arrangement would partially prevent oxidation of the lipids during the process since they are not exposed directly to the surrounding drying air. After dehydration, however, lipid constituents would not be effectively protected because both starch and cell wall in the dehydrated state would act only as spreading matrices for the lipid, thus leaving the newly-acquired structure of the granule more susceptible to oxidative attack.

Walter and Purcell (1974) observed that free and bound lipids in dehydrated potato oxidize at different rates, surface fatty acids being destroyed much faster than bound fatty acids. In agreement with this statement, Khan and Hadziyev (1979) found that the existence of lipid-protein combinations in the potato granule greatly minimize the breakdown of lipids. This implies that their oxidation rate would be, among other things, a resultant of the nature of the matrix with which the lipids are associated.

Pun (1979) reported a 14.7% loss of total lipids during the entire freeze-drying process; major losses (12.9%) occurring at the steam-cooking/hot mashing steps. Mainly the phospholipid fraction, was affected. Cooking and mashing caused only a slight decrease in the sum of polyunsaturates. The whole process slightly decreased the unsaturation ratio (1.4) of the raw potato. (UR: a ratio of the sum of linoleic and linolenic acids to that of palmitic and stearic acids). In light of these results, Hadziyev and Steele (1979) concluded that phospho- and glycolipids should be considered as potential major off-flavor precursors.

2. Off-Flavor Development

As demonstrated by Burton (1945,1949) and confirmed by Hendel et al.(1951), the two major deteriorative processes that affect the flavor quality of dehydrated potato products are non-enzymatic browning and oxidative rancidity. The former, which occurs during processing, produces flavor defects that arise from reducing sugar-amino acid interactions. The scorched flavor development induced by non-enzymatic browning reactions can be easily retarded by reducing moisture content (below the monolayer value), adding sulfite or lowering storage temperature. Sapers et al.(1972) pointed out that the shelf life of dehydrated potato products is usually limited by the development of hay-like off-flavors attributed to lipid oxidation rather than to non-enzymatic browning.

Although the lipid content is only 0.5% (dry weight) of the potato, its main constituents, considered as essential for maintening the integrity of membranes, are rich in polyunsaturated fatty acids and these in turn are highly susceptible to oxidative rancidity. Essentially, the degradation process occurs at the methylene interrupting conjugated systems in unsaturated glyceride molecules.

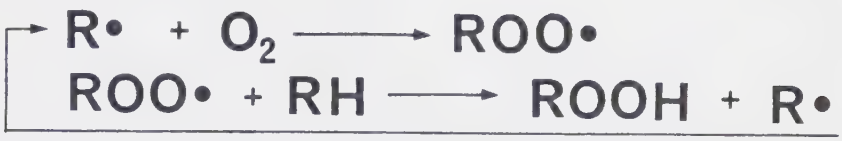
In general, autoxidative rancidity is regarded as the result of an initial oxygenation of unsaturated fatty acids followed by subsequent scission of the aliphatic carbon chain to yield volatile and odorous products (Swoboda, 1973). Mechanisms of autoxidative rancidity in fat, oil and model systems have been widely investigated (Labuza et al., 1969; Loury, 1972b; Sherwin, 1972, 1978; Uri, 1973). Results reported by these authors all support subdivision of the "free-radical" mechanism into three major steps, namely, initiation, chain propagation and termination. A generalized scheme for such rather complex autoxidation processes is presented in Figure 3.

Heat, light, trace metal and pigments have been reported to serve as catalysts in the initiation step (Uri, 1973). However, their exact roles and the kinetics of the reactions in which they are involved have still to be investigated; the initiation reaction being the least understood step in the formation of hydroperoxides.

The rate of lipid oxidation in dehydrated products is affected by not only its lipid composition but also by the

INITIATION reactions

PROPAGATION reactions



ADDITION or CROSS-LINKING

DECOMPOSITION of HYDROPEROXIDES

CHAIN SCISSION (formation of aldehydes)

TERMINATION reactions

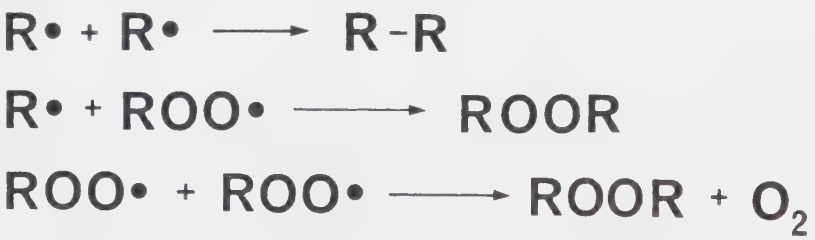


Figure 3. Schematic representation for autoxidation processes as proposed by Ranney (1979), where I or IH are species which form the free radicals R•, and RH is an organic substrate.

moisture content (Labuza et al., 1970), processing conditions (Anderson et al., 1963a; Sapers, 1975), surface area and other factors not as yet elucidated (Buttery, 1961; Fritsch and Gale, 1977).

Buttery et al.(1961) related off-flavor formation in air-packed potato granules to oxygen consumption and the loss of linoleic and linolenic acids during storage. A plot of oxygen absorbed from the headspace versus storage time gave a curve typical of lipid oxidation: an induction period followed by rapid oxidation, and a tailing-off period. The extent of autoxidation was monitored using the unsaturation ratio. Starting at 3.0 for freshly dehydrated granules, the UR decreased to 2.6 at the end of the induction period, to 1.7 at the end of rapid oxidation and to 1.2 during the tailing-off period. The volatiles from autoxidized granules corresponded to theoretically expected degradation products of linoleic and linolenic acids.

Buttery and Teranishi (1963) showed that the gas chromatographic determination of hexanal in the vapors of dehydrated potatoes suspended in boiling water was a measure of linoleate oxidation. Such a technique was proved to be more sensitive than peroxide determination, the thiobarbituric acid method or oxygen uptake. The concentration of hexanal in dehydrated potatoes has been shown to closely follow the development of off-flavors (Boggs et al., 1964). Recently, a study made by Fritsch and Gale (1977) used hexanal content as a measure of rancidity

in low fat foods. Fresh dehydrated potatoes contained less than 1 ppm hexanal. Whenever rancid odors were first noted in a storage test, the hexanal concentration was found to be between 5 and 10 ppm. Prior to the onset of rancid odors, no significant changes in the pattern of the chromatograms were noted except for an increase in the hexanal peak. Nevertheless, the authors emphasised that any attempt to define the quality of food from the measurement of a single compound such as hexanal would be presumptuous. However, it seems that such a procedure can provide valuable information when used in low fat dehydrated foods, such as potato granules. Research in this area is still in progress.

3. Antioxidants

a. General characteristics

Historically, it was observed as early as 1797 that oxidative reactions may be retarded by certain compounds (Berthollet,1797). Later, the basic chemical concepts of autoxidation and antioxidant effect were more fully explained by Moureu and Dufraisse (1925,1926). Since that time, considerable attention has been given to the development of antioxidants for varied applications, leading to the present structure of food additive regulations with lists of toxicologically acceptable and effective antioxidants (Boehm and Maddox, 1973).

An antioxidant should, ideally, be effective at low concentrations and should be non-toxic, tasteless, odorless,

stable and easily incorporated. For nearly 40 years, synthetic antioxidants have played an important part in preserving the quality of a variety of edible fats, oils and food products. The currently used compounds-primarily butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ)- are added directly to human food at a rate of about 3 million pounds per year (Waldrop, 1980).

Recently, a study to assess the safety of the so-called GRAS (Generally Recognized As Safe) substances was completed by the Select Committee of the Federation of American Societies of Experimental Biology (FASEB). The Committee recommended additional studies on the two most common food antioxidants, BHA and BHT, because of unresolved questions in research data. The FDA (Food and Drug Administration) usually issues interim regulations, requiring that certain safety tests be undertaken, meanwhile permitting continued use of the substances (Anon., 1981). An extensive study on the metabolic fate in animals of hindered phenolic antioxidants (Hathway, 1966), indicated that BHA was rapidly absorbed, metabolized and completely excreted, while BHT showed intermediate rates of absorption, metabolism and elimination from body tissues. In feeding tests, high levels of the antioxidants BHA, BHT, and TBHQ caused significant enlargement of the liver. BHT, but not BHA or TBHQ, also increased liver microsomal enzyme activity (Waldrop, 1980). Current research is focussed on efforts to manufacture

antioxidant molecules too large to be absorbed in the gastrointestinal tract (Weinshenker, 1980). A very promising polymer, called Anoxomer, has recently been synthetised, but it is not yet permitted for use in practice.

Most natural and synthetic food-grade antioxidants belong to a group of compounds known as "hindered phenols", in which the reactivity of the phenolic group is sterically hindered by substituents on the aromatic ring.

BHA consists of a mixture of 2- and 3-tert-butyl-4-hydroxyanisole. These compounds are readily synthetized by butylation of para-methoxyphenol. Since 3-BHA exibits greater antioxidant activity due to the difference in degree of steric hindrance of the phenolic group by the tert-butyl groups (Kauffman, 1977), it is desirable to obtain a high content of this isomer. Excellent separation of the two isomers has been carried out by two-dimensional thin-layer chromatography using benzene and acetonitrile (Sahasrabudhe, 1964), by gas-liquid chromatography equipped with a hydrogen flame ionization detector (FID) using diphenylamine as internal standard (Mahn et al., 1970) and on Sephadex LH-20 (Kauffman, 1977). As reported by Maga and Monte (1977), the 3- and 2-isomers are commercially produced in an approximate ratio of 96:4. BHA is practically insoluble in water and readily soluble in fats. This white, crystalline solid has been shown to possess good heat stability and excellent carry-through properties (Boehm and Maddox, 1973).

Since its introduction as a food additive in 1954, BHT has received much attention in various areas of research (Fulton et al.,1980). BHT is used for the manufacture of synthetic resins, rubbers, fuel gasolines as well as antioxidant of foods. Uemura (1978) reported that BHT is produced in Japan in the amount of 8000 tons annually. As a food antioxidant, BHT exhibits many properties similar to BHA. Where good carry-through is required, BHA is preferred, although BHT is cheaper (Boehm and Maddox,1973). In summary, phenolic antioxidants are suitable for use in food because they impart little or no color, have minimal taste and odor, are stable enough for a reasonably long shelf life, hold up at oven and fry pan temperatures, and are soluble in food oils.

b. Mechanism of Antioxidant Action

Since the chain reaction of fat and oil autoxidation is initiated and propagated by the formation of free radicals, removal or deactivation of the free radicals should terminate autoxidation in early stages before end products responsible for organoleptically detectable rancidity can form (Stuckey, 1962; Loury, 1972a; Sherwin, 1972). The function of an antioxidant structure is to provide a hydrogen atom to complete the electron structure of the free radical. The overall mechanism of antioxidant action, as proposed by Sherwin (1978), is illustrated in Figure 4. By replacing the fat molecule as the donor of hydrogen atoms,

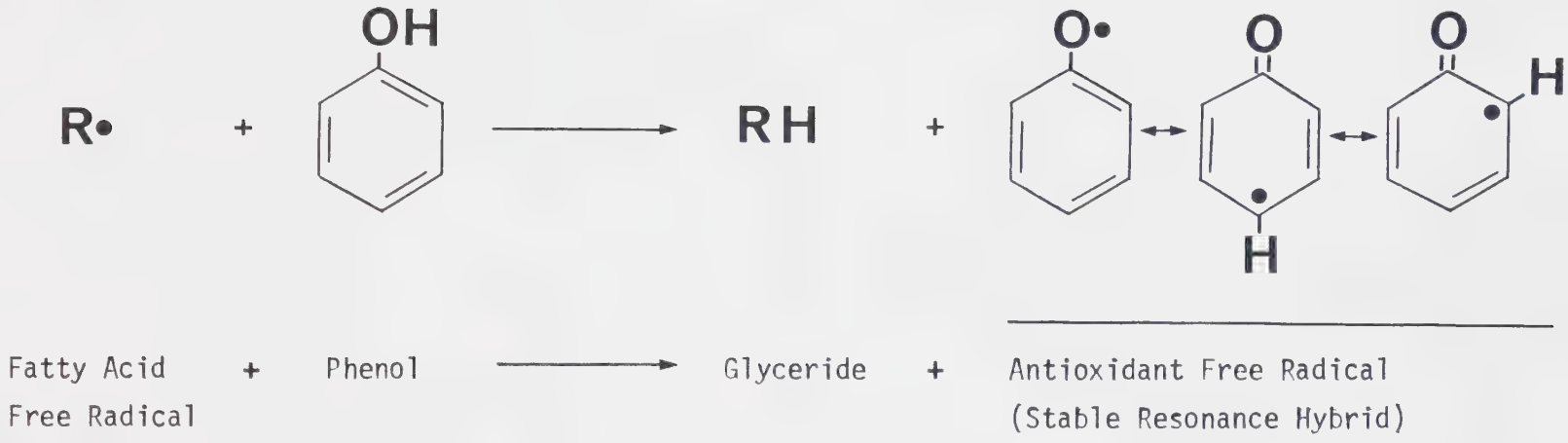


Figure 4. Schematic representation for phenolic antioxidant action mechanism as proposed by Sherwin (1978).

antioxidant terminates the chain reaction. Therefore, oxidative rancidity is retarded until the supply of antioxidant is exhausted. The loss of hydrogen by the antioxidant leaves the antioxidant itself a free radical. It would seem that this free radical could capture a hydrogen atom from another fat molecule and thus continue the chain reaction. However, it is believed that the antioxidant free radical, a semiquinone, assumes a more stable configuration by internal rearrangement of electrons. Because of this electron resonance, the semiquinone does not have the capability of initiating or propagating the oxidation reaction. Hence, these phenolic antioxidants are classified as primary antioxidants.

BHA and BHT both have a single hydroxyl group in their molecules, and they are sterically hindered phenols with tertiary butyl groups positioned ortho to the hydroxyl groups. BHT is hindered somewhat more than BHA, and this no doubt accounts for some differences in potency between these two compounds. There is considerable evidence that they will act synergistically when used in combination. That is, the combined effect of the two antioxidants is greater than the sum of the individual effects obtained when they are used alone. The synergistic effect has been attributed by Finkel'shtein et al (1977) to regeneration of the more active inhibitor at the expense of the less active inhibitor. Thus, when a BHA-BHT mixture is used, the inhibition of the oxidation process was due to the

interaction of BHA with peroxide radicals, and BHA is regenerated according to reactions in which BHT is involved.

Since heavy metals such as copper and iron play an important role in initiating or propagating autoxidation, the addition of chelates along with antioxidants has been shown to inactivate the pro-oxidant effects of such metals. These compounds which can complex heavy metal ions, are called secondary antioxidants. They have little effect in the absence of primary antioxidants. However, they do enhance or greatly prolong the action of primary antioxidants (Lundberg, 1966). Such synergistic ability has been ascribed to numerous compounds, such as phosphoric acid, lecithin, hydroquinone, methionine and citric acid (Kraybill et al., 1949; Stuckey, 1962). This phenomenon is often referred as "acid synergism" (Sherwin, 1972). An excellent review of the mechanisms of action was provided by Lundberg (1962).

c. Use of Antioxidants in Potato Granules

Maintenance of freshness in foods is a matter of utmost importance in their processing and marketing. In dehydrated potato products, although packaging in an inert atmosphere has often been proved to be quite effective in preventing oxidative deterioration (Stephenson et al., 1958; Drazga et al., 1964; Toffolo, 1968; Sapers, 1975), high costs of packaging and material, as well as the residual oxygen usually present (0.5-1.5%), strongly encourage the use of

antioxidants.

The role of phenolic antioxidants in potato granules is to prevent oxidative rancidity of the natural fat of the potato during shipment and storage. The amount allowed is subject to government standards. Guidelines for dehydrated potato granules permit for BHA or BHT or their mixture not more than 50 ppm (Canada), 10 ppm (USA), or 25 ppm for the U.K. and France. Talburt and Smith (1975) reported that a range of 1 to 5 ppm of BHA or 2.5 ppm of each antioxidant appeared to be desirable for use in potato granules.

Frequently, unsatisfactory performance of antioxidants in foods may be traced back to inadequacy of the application technique. As disclosed by Sherwin (1972), dispersing an additive in a food at a concentration of 100 or 200 ppm is a challenge even under the best of conditions. In the potato granule process, antioxidants are usually added at the mashing step. In order to obtain a good distribution in the mash, numerous methods of antioxidant addition have been investigated. Stuckey (1955) stressed the fact that since BHA and BHT are practically insoluble in water and added in extremely small quantities, a diluent must be used to ensure their complete distribution. An example is a patent assigned to the American Potato Co.(1967) in which an ethanolic solution of BHT was added to mashed potato prior to dehydration. Sherwin (1972) pointed out that, when BHA or BHT are added to complex food systems containing moisture, it may be necessary to develop emulsions or other types of

antioxidant solutions which are more compatible with the food product and which will serve to carry the BHA or BHT into the food. Along these lines, Sapers et al. (1975) suggested that antioxidants should be added as components of emulsions containing other ingredients or as alcoholic sprays since efforts to stabilize the membrane lipids in a mashed potato system must contend with the problem of dispersing fat-soluble antioxidants in a medium containing close to 80% water. The lack of ready access to sites of oxidation, i.e., polyunsaturated, polar lipids of the membranes was regarded by Hadziyev and Steele (1979) to be responsible of the poor protection in dried tissues such as potato granules. In the Add-Back process, antioxidants are usually applied with a formulated emulsifier added to cooked potatoes in the mash-mixing step. Some plants apply BHA as an ethanol solution and BHT as small crystalline beads (0.2-1.5 mm).

Steam distillation and volatilization, occurring mostly during the addition of antioxidants to the hot mash and during drying, cause dramatic losses. Preliminary results (Packer, 1981) indicated that the net retention of BHA in the Add-Back granules amounted to 40-50%, while for BHT only 2-5% of the quantity added initially was left in the final product. Thus, the end-product must be analysed for antioxidant content rather than depending on the quantity of antioxidant added as a quality control check. Additional losses of antioxidants during storage of dehydrated potato

products have been reported by Sapers et al.(1975) and Lisberg and Chen (1973). Earlier study made by Anderson et al.(1963b) postulated that the actual antioxidant content of a food will vary inversely with time elapsed since addition of the antioxidant.

In order to counteract difficulties in homogenizing antioxidants in the moist product as well as to avoid losses during processing, BHA and BHT have been successfully applied to the packaging material of cereals (Stuckey, 1955; Caldwell and Shmigelsky, 1958; Shmigelsky et al., 1964; Anon., 1977). Benefits of this technique are believed to derive from slow migration of the volatile antioxidants from the package into the food product or, where there is transfer of fat or oil from the food product to the packaging material, from inhibition of oxidation on the inner surface of the package itself (Sherwin, 1972). Based on these findings, oxidative rancidity in potato granules packaged in antioxidant-treated material should be monitored in order to establish whether or not antioxidants can be effective in such a system.

C. *Problems in antioxidant analysis*

An additional problem encountered during studies of the effectiveness of antioxidants is their quantitative analysis, since their concentration in the product is normally at the ppm level. Two general routes exist for the determination of antioxidants in foods (Stuckey and Osborne, 1965). One is for foods with a high fat content (such as nuts, processed meats, and some baked products) and is of no direct relevance to this study, while the other is for low-fat foods like some cereals, rice and dehydrated mashed potato products. As outlined by Hadziyev and Steele (1979), the determination of phenolic antioxidants in potato granules, though assumed to be standardized, is still subject to further improvement.

1. Extraction Procedures

Techniques involving solvent extraction and/or steam distillation are employed to separate antioxidants from food. Only a few of these techniques are suitable for dehydrated potato products. Filipic and Ogg (1960) and Sloman et al.(1962) isolated BHA and BHT from dehydrated potato samples by a rapid steam distillation procedure at atmospheric pressure.

Buttery and Stuckey (1961) extracted antioxidants from add-back potato granules by soaking the samples overnight in petroleum ether. This method was quite time consuming. Cold solvent extraction was also used by Schwecke and Nelson

(1964), who percolated diethyl ether through a chromatographic column containing potato granules.

More recently, a systematic study of the problems encountered in analysis of BHA and BHT in dehydrated products was disclosed by Bieth et al.(1978). The authors reported that the accuracy of the determinations appears to rely heavily on the extraction method used to isolate antioxidants from the dehydrated mashed potato products. Efficiencies of extractions by steam distillation and by petroleum ether (cold or hot) were compared in order to establish the best extraction procedure. As results using these methods varied widely, extractions were tried with superheated steam, carbon disulphide (cold or hot), ethanol (cold or hot), and continuous extraction using a Soxhlet apparatus. The best extraction rates were obtained by continuous extraction using ethanol (96%) on previously hydrated material. This procedure was found to be simpler than the steam distillation method of Filipic and Ogg (1960). Nevertheless, Bieth et al.(1978) strongly recommended both techniques as standard methods of determining BHA and BHT in dehydrated mashed potato products in place of the procedure of Takahashi (1970). The latter has been used as a standard technique to determine BHA and BHT in fats or cereal-based products but did not appear to be satisfactory for dehydrated potato products.

To further complicate the matter, results of extraction of antioxidants in commercially dehydrated potato products,

where antioxidants were incorporated during the process, differed much from those dehydrated potato products where known quantities of BHA and BHT were added after processing to spike antioxidant-free samples.

Accordingly, Bieth et al.(1978) stressed the fact that spiking dehydrated potato products with known quantities of antioxidants prior to extraction would not accurately assess the effectiveness of an extraction technique even if total antioxidant quantities were recovered. As a result, doubt was cast on previous extraction studies in which mixtures of dehydrated potato products and antioxidants had been prepared in the laboratory (Filipic and Ogg, 1960; Buttery and Stuckey, 1961; Sloman et al., 1962; Schwecke and Nelson, 1964; Takahashi, 1970).

Moreover, by conducting freeze-drying investigations, Bieth et al.(1978) proved there was some form of linkage between the antioxidants and the potato matrix during the dehydration process. They emphasized the need for hydration of the dehydrated potato sample prior to extraction (by adding water in an approximate ratio of 1:1 w/w) in order to break the undefined linkage.

Despite the above studies, the exact nature of the retention of antioxidants in potato granules has not been fully elucidated, and further research in this area was needed in order to improve the existing extraction techniques.

2. Determination procedures

The widespread use of phenolic antioxidants in food products has stimulated the development of numerous techniques for their determination. Quantitation has been accomplished by spectrophotometry, paper chromatography, thin layer chromatography, spectrofluorometry, gas-liquid chromatography , voltammetry and high performance liquid chromatography. Since the subject has been critically reviewed by Stuckey and Osborne (1965), by the Eastman Kodak Company (Anon.,1967) and, more recently, by Halot (1971), emphasis in this discussion will be given largely to literature pertinent to the determination of antioxidants in dehydrated potato products.

a. Spectrophotometry

Filipic and Ogg (1960) colorimetrically estimated BHA and BHT in the steam distillate of dehydrated potato products. BHA was determined by reaction with 2,6-dichloroquinonechloromide (Gibbs, 1927). This reaction is specific for BHA in the presence of BHT because the reactive sites ortho and para to the phenolic hydroxy group in BHT are blocked. Then the total antioxidant content was measured by Emmerie-Engel's method (Emmerie and Engel, 1938). The basis for the color production is the reduction of ferric chloride to the ferrous state by the antioxidants leading to the formation of an orange-red iron-bipyridyl complex absorbing at 515 nm. Substraction of the BHA

contribution, as determined by Gibbs reagent at 620 nm, gave the amount of BHT.

Halot (1971) reported that the method of Filipic and Ogg (1960) was nonspecific since the presence of other reducing substances may give rise to overestimation of antioxidant levels. Along these lines, Filipic and Ogg (1960) had observed that sodium sulfite, added as a browning inhibitor, will interfere with both colorimetric methods of analysis. The detection limit for BHT (BHA) was found to be 10 ppm.

Sloman et al.(1962) combined the Gibbs reagent method for BHA and the dianisidine method (Szalkowski and Garber, 1962) for BHT in an overall scheme for analysis of these two antioxidants in dehydrated potatoes as low as the 5 ppm level.

Colorimetric reactions in general are excellent for BHA and BHT analyses in food. However, they are not completely specific. For example, 2,6-dichloroquinonechloromide will react with any phenol with a vacant ortho or para position, not just with BHA. Also, ferric chloride-2,2'-bipyridine will give color with any phenol.

Worthy of note is that none of the published spectrophotometric methods allow a simultaneous determination of BHA and BHT (Senten et al., 1977). This is only possible with GLC, voltammetry and HPLC.

b. Gas-Liquid Chromatography

Since its development in the 1950's, GLC has become one of the more powerful analytical tools. One major advantage is that detection of very small quantities of material is possible. Buttery and Stuckey (1961) described direct extraction of BHA and BHT from potato granules followed by GLC of the eluate on an Apiezon L column using flame ionization detection. A range of 0.5 ppm to 10 ppm of antioxidant was analysed, with an average error of less than 11%. This method, although sensitive, required aging of the column for 1 week at 220°C. Furthermore, potato lipids, which were extracted by petroleum ether along with the antioxidants, produced some base line noise, thus limiting the sensitivity at which the detector could be used. However, the main error seemed to result from incomplete extraction of antioxidants from the granules.

Schwecke and Nelson (1964) introduced the use of 3,5-di-tert-butyl-4-hydroxyanisole (Di-BHA) as an internal standard in the GLC assay. Unknown amounts of BHA and BHT were determined by comparison with a known concentration of Di-BHA in the same sample. This procedure obviates having to measure carefully the amount of a standard solution. These authors analysed in the range of 5-30 ppm antioxidant using a Silicone Gum SE-30-Tween 80 column and flame ionization detection. The method was proven to be sensitive, accurate and rapid.

Since contamination of the column by potato lipids may be a problem in analysis by GLC, Hartman and Rose (1970) suggested that a short precolumn of siliconized glass wool be located in the sample injection port as a trap for nonvolatilized lipids. The precolumn had to be cleaned daily.

Takahashi (1970) described a GLC method for BHA and BHT in breakfast cereals, using Di-BHA as the internal standard. Bieth et al.(1978) used this method in studying the analytical problems of antioxidants in dehydrated foods.

GLC has recently made remarkable progress. This was well illustrated when BHA and BHT along with 10 other preservatives were analysed by GLC after steam distillation into methylene chloride by Isshiki et al.(1980).

c. Voltammetry

The early voltammetric investigations started when McBride and Evans (1973) developed a linear-sweep voltammetric method for the determination of sterically hindered phenolics, including BHA, BHT, PG, tocopherol and tocopheryl acetate. The procedure permitted rapid analysis of phenolic antioxidants in fat and tocopherols in food, from a single voltammogram. A voltammetric cell was fitted with a saturated calomel electrode (SCE) as the reference electrode; a platinum wire as the so-called counter electrode, and, a glassy carbon electrode (GCE) as the working electrode.

In order to ensure reproducibility and high sensitivity, a standard pretreatment procedure was applied before each voltammogram. The glassy carbon electrode was rinsed, then its planar surface was buffed for about five seconds using a rotary polisher and alumina powder. This was followed by an ethanol rinse, and insertion into the cell. The next sample solution was allowed to become quiescent for at least 30 seconds before recording the voltammogram. Recently, Van Der Linden and Dieker (1980) confirmed that glassy carbon electrode need some kind of pretreatment to obtain reproducible results.

An organic solvent mixture of ethanol and benzene was used by McBride and Evans (1973). Though numerous electrolytes ranging from potassium hydroxide to nitric acid were investigated, dilute sulfuric acid seemed most generally useful. Thus, 0.12 M sulfuric acid in ethanol-benzene (2:1 v/v) was established as the best supporting electrolyte solution.

Despite the rapidity of the method, McBride and Evans (1973) suggested the use of pulse voltammetry to improve sensitivity since linear-sweep voltammetry could not detect traces (sub ppm) of naturally occurring tocopherols or synthetic antioxidants.

Analytical evaluation of Differential Pulse Voltammetry (DPV) at stationary electrodes was carried out by Rifkin and Evans (1976). Results demonstrated that analyses of mixtures could be achieved when the peaks of the oxidizable

substrates were well separated. Consequently, BHA and BHT were successfully determined in the presence of large concentrations of one another. The voltammogram showed two separated peaks, with the height of the more positive peak (BHT) being unaffected by the presence of the more readily oxidized component (BHA). This was of most importance to verify since in DPV the total current at the more positive peak is due to oxidation of both components.

Differential Pulse Voltammetry was applied succesfully by Haydar and Hadziyev (1979) in the analysis of BHA and BHT in dehydrated potato granules. After spiking the samples with known amounts of antioxidants, a rapid extraction was performed using benzene as solvent. BHA and BHT were accurately determined since their wave potentials were well separated; BHA, +0.78 V and BHT, +1.03 V.

More recently, Brieskorn and Mahlmeister (1980) effected the qualitative and quantitative determination of single phenolic antioxidants, in lipids and food and of tocopherol in human blood by DPV on a rotating GCE. The sensitivity increased by over 100% compared with the static electrode when a rotation speed of 70 revolutions per second was used. Under these conditions, only a slight positive shift in peak potentials of BHA (BHT) and some natural phenolics was observed.

3. Possible interference from natural potato phenolics

Buttery and Stuckey (1961) stated that many naturally occurring phenolic materials in potatoes interfere with analysis of BHA and BHT by colorimetric methods unless a tedious steam distillation procedure is used. Steam distillation procedures, although time consuming, apparently remove most interferences in the colorimetric analyses of BHA and BHT since natural potato phenolics either are not steam distillable or are decomposed at the temperatures reached during distillation. Nevertheless, as in UV analyses, it is more reliable when the food containing no antioxidant can be analysed simultaneously as a blank to determine if interferences exist.

Voltammetry with linearly varying potential (McBride and Evans,1973) has proven satisfactory in separating the hindered phenolic compounds, except for β- and γ-tocopherol, which were superimposed. On a single voltammogram, the peaks potentials (in Volts) were: α-tocopherol, +0.57; β- and γ-tocopherols, +0.67; δ-tocopherol, +0.74; BHA, +0.78; and BHT, +1.03. The peak potential of BHA practically coincided with that of δ-tocopherol, hence, BHA can only be determined if samples do not contain appreciable amounts of this tocopherol isomer. Similar results were obtained by Podlaha et al.(1978), Haydar and Hadziyev (1979) and Brieskorn and Mahlmeister (1980) using Differential Pulse Voltammetry. Generally, potatoes do not contribute a significant amount of tocopherols to the diet. A search by GLC for tocopherols

in potato lipid unsaponifiable matter showed that neither α-, β- nor γ-tocopherol was present in appreciable quantities (Lepage, 1968).

Tocopherol and carotene isomers were not detected in granules of the potato cv. Netted Gem (Haydar and Hadziyev, 1979). Also, other natural potato phenolics did not interfere in the BHA (BHT) voltammetric assay.

In conclusion, the simplicity, ease and speed of carrying out determinations, and the accuracy and reproducibility of Differential Pulse Voltammetry are factors that indicate its usefulness in assaying phenolic substances. Based on the above, DPV was chosen as the method in this study for simultaneous determination of BHA and BHT in dehydrated potato granules.

III. EXPERIMENTAL

A. *Potato Tubers*

Cv. Netted Gem (Russet Burbank) with specific gravity of 1.096±0.002, corresponding to a dry matter content of 25.0 %, was grown in Southern Alberta and supplied by "Iwabuchi and Sons Ltd.", Edmonton.

The tubers were stored at 4°C and were reconditioned at room temperature for 10 days before use. Proximate analysis gave the following percentages on a dry matter basis of peeled tubers: crude protein 8.8 (N x 6.25); lipid 0.5; crude fiber 2.5; ash 4.0; and starch 75.0. Amylose content of the starch was 21.2 %, as determined by potentiometric titration with iodine (Chung and Hadziyev, 1980).

B. *Chemicals*

Pectin (Citrus pectin), with a 55-60 % esterification degree and a mol. weight range of 150-300 x 10^3, was from ICN-Nutritional Biochemicals, Cleveland, OH.

Starch amylose of 273-275°C m.p. (decomposition) with a mol. weight over 150,000, and amylopectin, both from potato, were supplied by Aldrich Chem. Co. Inc, Milwaukee, WI, and by BDH Lab Chemicals Division, Toronto, Ont.

The C16 type of emulsifier containing at least 90% distilled monoglycerides (with at least 90% of 1-isomer, the rest being 2-isomer) derived from hydrogenated palm oil which had been enriched with palmitic acid was from Vauxhall

Foods Ltd., Vauxhall, Alberta.

All other chemicals used in this study were of reagent grade and were supplied by Fisher Scientific Co.

C. *Equipment*

Centrifuges used were: International Centrifuge, Size 2 (International Equipment Co., Boston, MA), and Beckman Model J21B Refrigerated Centrifuge (Beckman Instr. Inc., Palo Alto, CA).

The Polarised-light microscope was a Zeiss-Winkel, made in Germany.

The Freeze-Dryer used was: RePP Freeze-Dryer, manufactured by Virtis Co., Inc., Gardiner, NY.

The Polarographic Analyser, Model 174A, was used in conjunction with a Houston Omnigraphic X-Y Recorder, Model 2200-3-3. The Saturated Calomel Electrode (#9311); the Glassy Carbon Electrode (#9333); the Polishing Kit (#9320) and the Pyrex glass voltammetric cell with Pt wire Counter Electrode, were all supplied by Princeton Applied Research Co., Princeton, NJ.

The equipment used during processing of freeze-thawed potato granules were:

Abrasive potato peeler, Model 6115, Vegetables Slicer, Model H 4212, Kitchen Aid Mixer equipped with a flat beater, all three supplied by The Hobart Mfg. Co., Don mills, Ont.

Stainless steel trays (60 x 46 cm).

Air blast freezer with minimum air temperature of -29°C and

air velocity of 1.42 m^3/s.

Manestry Petrie Fluid-bed Dryer, Model MP.10.E., equipped with a stirrer as designed by Ooraikul, (1973).

Manestry Machines Ltd., Speke, Liverpool, England.

Speedomax 12 point temperature recorder, Leeds Northrup (Canada Ltd).

Canadian Standard Sieve series, and portable Sieve shaker, the W.S.Tyler Co. Ltd. Ste-Catherine, Ont.

Other equipment used:

Forced draft iso temp oven from Fisher Scientific Co., Ltd.

Büchi Rotavapor Büchi Glasapparate Fabrik, Flawil, Switerzland.

Hydraulic press, F. S. Carver, Inc., Summit, NJ.

Kitchen-aid mixer, Braun AG, Frankfurt, West Germany.

Virtis Homogenizer Model 45, Virtis Research Equipment Co., Gardiner, NY.

D. *Methods*

1. Major Potato Constituents

a. Isolation of Cellulose from whole tuber

The tuber cell wall preparation was used as a cellulose matrix. It was isolated from batches of dried tubers mechanically disintegrated in ice-cold water (containing 500 ppm Na-sulfite) using a Virtis homogenizer at full speed. The slurry was then squeezed through Miracloth and washed extensively with deionized water until the residue was free of starch when examined under the polarized-light microscope (Moledina et al., 1978a).

b. Isolation of Protein from whole tuber

Crude proteins were obtained from potato sap extruded from peeled and diced tubers. The dices were wrapped in a cotton cloth and subjected to a pressure of 25 x 10^3 p.s.i. using a hydraulic press. The sap was collected, then the cake was dispersed in 10 % aqueous NaCl and pressed again. The combined saps were diluted in water and the protein was precipitated at 70°C for 30 min. The fluffy coagulate was collected by low-speed centrifugation and then washed with acetone and ethyl ether. The protein, slightly greyish in color, was air-dried, ground into powder in a mortar with pestle and stored at -20°C.

c. Isolation of Native Starch grains from whole tuber and preparation of Gelatinized Starch

Potato starch was isolated from peeled and diced tubers which were homogenized in ice-cold water (containing 500 ppm Na-sulfite) using a Waring Blendor at low speed. The slurry was squeezed through a 100-mesh polyester sieve cloth and the filtrate centrifuged at 700 x g for 10 min. The supernatant and the amber-brown protein layered on the starch sediment were removed, and the starch was resuspended in water and recentrifuged. This purification step was repeated until no protein and cell debris were evident under a polarized-light microscope. The final product was treated with ethanol, ethyl ether and acetone, and air-dried.

A batch of the above preparation was used to obtain gelatinized and retrograded starch. Gelatinization was performed at 70°C for 20 min with 100 g starch suspended in 1 L demineralized water. The gel obtained was cooled to 4°C, the water decanted and the sediment frozen at -25°C for 3 h. The frozen gel was then freeze-dried and ground to a powder in a Waring Blendor at high speed.

d. Antioxidant Incorporation into Major Potato Constituents

The procedure used is as described by Kirleis and Stine (1978). In order to assay the strength of antioxidant binding to potato constituents, impregnated matrices were prepared by freeze-drying. An ethanolic antioxidant solution

(100 ppm of BHA on dry weight of the potato matrix) was added to an aqueous slurry of a potato constituent (protein, starch, etc). Uniform dispersal was achieved with a Kitchen-aid mixer run at low speed for 5 min. Portions of the slurry, 40-60 g, were then transferred into plastic containers and freeze-dried at a condenser setting of -50°C and a shelf temperature of 25°C using a Virtis model RePP sublimator. Samples were taken after each 0.5-1 h and analyzed for antioxidant and moisture contents.

e. Moisture Content

Moisture content on major potato constituents and potato granules were determined from the average weight loss from three replicates after heating 1 g of each at 105°C for 4 hr in a laboratory oven.

2. Preparation of Antioxidant (BHA) - Amylose Inclusion Compound

The procedure used is as described by Osman-Ismail (1972). Potato amylose corresponding to 1.5 g dry matter was suspended in 15 ml of distilled water, treated with 15 ml of 0.5 N NaOH and mixed until the suspension solubilized. The amylose solution was adjusted to pH 6.5-6.8 with 0.5 N HCl and then 10 mM K-phosphate buffer pH 7.0 was added (5% v/v). The solution was diluted to 1% with water, heated at 100°C for 1 h, then cooled to 70°C and deaerated with nitrogen. Under the nitrogen stream, BHA was incorporated (25 % on

amylose, dry matter basis) and the temperature of the mixture was then gradually lowered to 25°C over 24 h in a water bath. The precipitated amylose-BHA complex was collected by centrifugation at 6000 x g for 15 min, freeze-dried and stored at -20°C.

3. X-ray Diffraction Analysis

Diffraction patterns of amylose-BHA inclusion compounds were recorded on a Philips Model PW-1011-60 diffractometer equipped with a curved crystal AMR monochromator. Copper Kα radiation (1.5418 Å) was used with a time constant of 4 sec, an angular scanning velocity of 1° 2θ and a chart speed of 1 cm/min.

4. Antioxidant Extraction

Dehydrated major potato matrices were extracted by a continuous Soxhlet procedure using 95% ethanol as solvent (Bieth et al., 1978). As emphasized by the authors, rehydration of the sample (ratio 1:1 w/w) had to be performed prior to the extraction.

5. Antioxidant assay by Differential Pulse Voltammetry

A Princeton Applied Research, Model 174A polarographic analyzer was used in differential pulse mode (Brieskorn and Mahlmeister, 1980) in conjunction with a Houston X-Y recorder. Operating conditions were as follows: potential scan rate 2 mV/sec; scan direction +, with a range of 1.5 V

for BHA and 3.0 V for BHT and an initial potential of +0.3 V; sensitivity for a full scale recording 50 μA; low pass filter off; and a 50 mV pulse applied for 56.7 msec on the normal voltage ramp.

A glassy carbon electrode vs SCE was used in addition to a Pt-wire counter electrode. In order to obtain reproducible results, the electrode surface was repolished between each voltammogram on a felt cloth tissue wetted with γ-alumina slurry.

For voltammetric assay, the ethanol antioxidant extract was evaporated at 30°C to below 10 ml. Then the volume was adjusted to 10 ml with ethanol and transferred to the voltammetric cell. The extract was diluted with 5 ml of benzene to reach the solvent ratio of 2:1 ethanol-benzene, and 1.5 ml of the electrolyte (0.12 M sulfuric acid in ethanol-benzene, 2:1 v/v) was added to make a total volume of 16.5 ml.

Anodic waves were recorded at room temperature. Quantities of antioxidant in the samples were calculated as follows:

(1) The peak height (cm) was expressed in ppm (or in μg/ml in the cell) via the best least squares fit coefficients of the regression equation calculated from the phenolic compound standard curve.

(2) Then, the ppm value was multiplied by the sample dilution factor (16.5) in order to obtain the total amount of antioxidant (μg) in original 10 ml ethanol extract.

(3) Finally, the concentration of antioxidant in ppm (dry matter basis) was obtain by dividing the total quantity (μg) by the dry weight (g) of the sample extract.

E. *Production of Potato Granules*

1. Potato granule production with an Add-Back process

All granules used in this study were Add-Back granules supplied by Vauxhall Foods Ltd., Vauxhall, Alberta. The method used consisted of steam-peeling, trimming, slicing, washing, precooking, cooling, steam-cooking, mashing by addition of dried A-B granules, conditioning, remixing, air-lift drying, fluidized bed drying, cooling and sifting. Incorporation of antioxidants (as ethanolic solution for BHA and small crystalline beads for BHT) was done at the mashing step along with additives such as sodium acid pyrophosphate, sulfite and C16 type of emulsifier.

2. Potato Granule Production with a Freeze-Thaw process

The procedure used is as described by Ooraikul (1973). The granule process consisted of the following steps: peeling, steam-cooking at atmospheric pressure, mashing, freezing at -29°C for 3 h in an air-blast freezer, followed by thawing at room temperature, and predrying in a semi-pilot scale fluidized bed drier. In the mashing step, 100 ppm (wet weight) of BHA were added along with other additives as in the Add-Back process.

F. *Antioxidant Determination in Potato Granules*

1. Antioxidant Extraction

Pet. ether (b.p. 40-60°C) was the extraction solvent when GLC assay was applied, while benzene was chosen for voltammetry since it was a component of the supporting electrolyte.

The procedure, lasting close to 30 min, involved extraction of the fully rehydrated samples with organic solvent, followed by removal of water and concentration of the organic phase containing the antioxidant.

Potato granule samples of 5 g were rehydrated at room temperature with 25 ml water for 10-15 min on a fritted glass funnel (diameter 7.0 cm ; pore size 25-50 μm). After removing the excess water by suction, the cake was mixed with 3 x 10 ml solvent and filtered each time. Rehydration (for 2 min) and solvent extraction were repeated. The extract was transferred to a 125 ml separatory funnel, shaken gently and left to stand 5 min. If needed, 10 ml of a 10% sodium chloride solution were added to break the emulsion. The organic layer was then concentrated on a rotary evaporator at 30°C.

Prior to GLC-assay, the initial solvent extraction step was preceded by addition to the cake of a 1 ml solution containing internal standard and a keeper. A stock solution, containing internal standard (62.5 mg of DiBHA) and a keeper (1 g of paraffin oil) in 25 ml pet. ether, was kept in a freezer. Fresh working solutions were prepared

daily by diluting 1 ml of stock solution with 25 ml pet. ether.

2. Gas-Liquid Chromatography assay

For GLC assay, the pet. ether extract was evaporated just to dryness. The residue was dissolved in 2 ml of carbon disulfide, and transferred to a sealed reaction vial. Aliquots of 4-5 μl were injected into a Varian model 3700 gas chromatograph equipped with FID detector and a 6 ft x 1/8" stainless steel column packed with 3% OV-17 on Gas Chrom Q. The runs were performed isothermally at 165°C with nitrogen (30 ml/min) as a carrier gas. Injector and detector were 200 and 220°C, respectively. Retention times were 4.8 min for BHT, 5.8 for BHA and 10.0 for DiBHA. A peak with retention time of 4 min was observed with BHT when evaporated extracts provided a yellow-brown instead of a colorless residue. The peak was observed when keeper addition was omitted in the granules extraction step.

3. Differential Pulse Voltammetry assay

For DPV assay, the benzene extract was concentrated to below 5 ml on a rotary evaporator at 30°C. Then, the volume was adjusted to 5 ml with benzene and transferred to the voltammetric cell. The extract was diluted with 10 ml of ethanol to reach the solvent ratio of 2:1, v/v. All subsequent operating conditions were as in DPV assay for antioxidants in major potato constituents.

4. Determination of naturally occurring antioxidants in potatoes

a. Natural Potato Phenolics Extraction

Potato granule samples of 2 g were rehydrated at room temperature with 8 ml of hot water (80°C) for 15-20 min on a fritted glass funnel (diameter 7.0 cm; pore size 25-50 μm). After removing the excess water by suction, the cake was mixed with 2 x 30 ml hot ethanol (80°C) and filtered each time. The ethanolic extract was then concentrated to 4-5 ml on a rotary evaporator at 30°C and adjusted to 10 ml with 95% ethanol prior to voltammetric assay.

b. Natural Potato Phenolics Measurement

For quantitative analysis, an aliquot of 5 ml of the ethanolic extract was transferred into the voltammetric cell. Then, the supporting electrolyte composition was made up as for BHA and BHT assay, and the voltammogram recorded. Samples were diluted when necessary to permit analysis in the central region of the standard curves.

Polarographic settings were identical as for synthetic antioxidant assay, except that scanning ranges of +0.3 to +1.8 V and +0.3 to +3.0 V were suitable for chlorogenic acid and tyrosine, respectively.

IV. RESULTS AND DISCUSSION

A. *Antioxidant Recovery from Freeze-Dried Matrices*

In a series of freeze-drying assays, it was found by analysis of variance that moisture removal from matrices (at moisture contents of 65 % or lower) had no significant influence on the extent of BHA retention. As illustrated in Figure 5, at a 63.2 % moisture level, amylose retained an average of 96.4 % BHA, a value which did not change when the moisture content was lowered to 33 or 4.2 %. On the other hand, amylopectin at 63.2 % moisture lost 20 % of BHA, but no further loss was observed with removal of more moisture. Similarly, the 86 % BHA retention with native starch, achieved after half of the moisture was removed, did not change during the rest of the drying process. The small 5 % BHA loss at a matrix moisture content of 50 % and the 1 % BHA loss for each additional 10 % removal of moisture suggest the strong affinity of BHA towards potato protein.

However, pectin matrix showed a 20-25% variation in BHA retention, because, due to its highly hydrophilic nature, the pectin-BHA slurry was freeze-dried for 10 hours more than the six other matrices. This result agrees with those of Kirleis and Stine (1978) that losses of BHA from a food model system increased substantially when the freeze-drying time was lengthened.

The means of the sums of all BHA recoveries found for each matrix at different moisture content levels were then

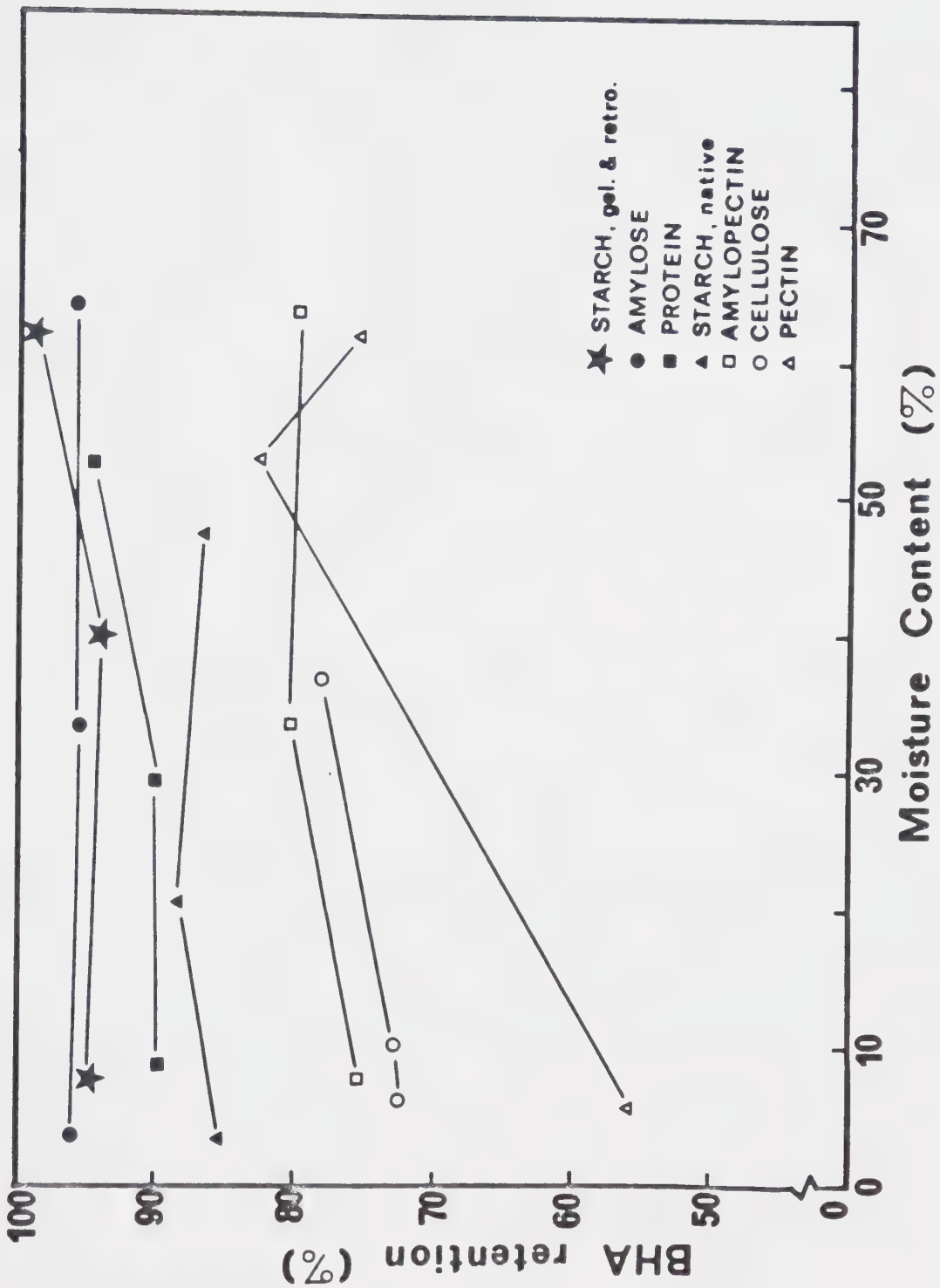


Figure 5. BHA retention by major potato constituents during the freeze-drying process.

used for further comparisons using statistical tools. As seen from Table 1, BHA retention depended on the nature of the matrix, and varied from 71.4-96.3 %. The explanation for these high retention values (well above the 40-50 % retention in the A-B process), probably lies in the fact that the freeze-drying process involves ice crystal sublimation rather than steam distillation and volatilization. In order to find out if there were any significant differences between the matrices, statistical analyses were carried out using the Student-Newman-Keuls Multiple Range Test procedure at the 95 % level (Steel and Torrie, 1980). This led to the matrices being divided into two subsets.

Selecting a matrix from each subset, the affinity of the antioxidants towards potato amylose (Subset I) and cell wall cellulose (Subset II) was tested by using solvents of increasing dielectric constant. Results are presented in Table 2. As seen for amylose in a solvent polarity range of ϵ=1.89 (pet. ether) to 24.3 (ethanol), the extent of BHA released was independent of solvent polarity and amounted to an average of only 3.5-4.3%. The inability of solvents of increasing polarity to extract BHA from amylose suggested that BHA is either entrapped within the realigned molecular network or bound within its helix in the form of an inclusion compound.

However, high recoveries were found with cell wall cellulose as a matrix, being close to 90% for low polarity

Table 1. Retention of BHA by Major Potato Constituents During Freeze-Drying

	Matrix	BHA Retention (%)[a]
I[c]	Starch, gelatinized and retrograded	96.3 (2.6)[b]
	Amylose	95.7 (0.6)
	Protein	91.4 (2.9)
	Starch, native	86.0 (3.2)
II	Amylopectin	78.2 (3.9)
	Cellulose	73.9 (3.1)
	Pectin	71.4 (14.7)

[a]Mean of the sum of all BHA recoveries found at different moisture levels.

[b]Standard deviation, n = 3.

[c]I and II are subsets calculated from the Student-Newman-Keuls' Multiple Range Test procedure.

Table 2. The Extent of BHA Recovery from Some Potato Constituents by Using Solvent Systems of Increasing Polarity

Solvent	Polarity (Dielectric Constant) ε	BHA Recovery, %			
		Amylose		Cellulose	
		With Water[a]	Without Water	With Water[a]	Without Water
Petroleum ether	1.89	37.6 (5.2)[b]	4.3 (1.8)	87.0 (11.8)[c]	88.1 (11.8)
Benzene	2.60	40.6 (2.9)	4.3 (0.8)	95.0 (12.2)	90.0 (11.9)
Ethyl ether	4.34	59.5 (2.8)	3.5 (1.1)	93.3 (13.9)	102.4 (16.8)
Acetone	20.70	95.6 (1.0)	4.1 (0.7)	102.4 (13.8)	98.4 (11.7)
Ethanol	24.30	96.5 (5.1)	4.3 (1.1)	105.7 (12.5)	96.9 (11.3)

[a]Hydration ratio 1:1 w/w, time 10 min.

[b]Values in parentheses are standard deviations, n = 3.

[c]Values in parentheses are estimated errors calculated from standard curve interval estimators.

and 100% for high polarity solvents. The values in parentheses are estimated errors calculated from standard curve interval estimators (Baird, 1962; Bowker and Lieberman, 1972). This procedure was required in order to determine a standard deviation value when, due to a limited quantity of freeze-dried cellulose samples, single rather than duplicate extractions were performed. Consequently, these estimated errors are the best confidence limits that can be calculated from the available information. Their high values are due to the "conservative approach" utilized in the calculations.

Most importantly, the results of Table 2 also indicate that, when amylose was hydrated for 10 min in a ratio of 1:1 w/w, antioxidant recovery increased. Low polarity solvents brought about close to a 40% BHA recovery, medium polarity 60% and high polarity up to 96%.

B. *X-ray Diffraction*

When BHA was added to solubilized amylose following the Osman-Ismail procedure, rapid precipitation occurred. This strongly suggested the formation of an inclusion complex. The X-ray diffraction patterns of host and guest compounds, their complex and an amylose matrix tenaciously holding BHA (95 ppm) are given in Table 3. As seen from interplanar spacings, pure amylose was characterized by three strong intensity spacings between 3.93-5.83 Å. Similar spacings

Table 3. Interplanar Spacing[a] and Intensity[b] Values from X-Ray Diffraction Patterns of Potato Amylose, BHA and Their Complex

Potato Amylose	BHA		Potato Amylose		
			Not Solubilized + BHA	Solubilized + BHA	
5.83 s	14.25 s	17.68 m	5.83 s	6.81 s	11.63 m
5.21 s	12.81 s	9.82 m	5.21 s	5.04 s	3.98 m
3.93 s	6.37 s	7.25 m	3.93 s	4.93 s	3.67 m
	5.98 s	6.66 m		4.75 s	
	5.79 s	4.93 m		4.67 s	
	4.25 s	4.72 m		4.65 s	
		4.02 m		4.48 s	
		3.75 m		3.25 s	
		3.63 m		2.73 s	
		3.56 m			
		3.04 m			
		2.07 m			

[a]Interplanar Spacings are in Å.

[b]Intensities are designated as strong (s) or medium (m).

were found in the unsolubilized freeze-dried amylose matrix with BHA. On the other hand, spacings of the inclusion complex were numerous in the range of 2.73-11.63 Å, and were mostly strong in intensity and differed significantly from those of pure crystalline BHA. These data substantiated evidence that amylose contains entrapped rather than inclusion clathrate forms of BHA, and indicated the importance of hydration, since it appears necessary to separate the associated amylose chains and release the entrapped antioxidants. It is important to note that cold water hydration would have been of no help in destroying the strong affinity of an amylose-BHA inclusion complex or in reversing retrogradation when using the commercial (and retrograded) amylose product.

c. *Antioxidant Extraction from Potato Granules*

The results obtained on matrices were confirmed on samples of dehydrated granules. As seen from Table 4, 8.8-9.5 ppm of the bound antioxidant were recovered from granules (moisture content 7.0 %) hydrated to 1:5 w/w (granules:water) and then eluted at room temperature with 30 ml of solvent. The poor performance obtained without rehydration was, again, clearly illustrated.

Elution experiments were carried out in order to estimate the exact volume of solvent required for total antioxidant extraction. As seen from Table 5, repeated hydration followed by additional extraction with 30 ml

Table 4. The Effect of Hydration of Potato Granules on the Extent of BHA Recovery by Solvent Systems of Increasing Polarity

Solvent	Polarity (Dielectric Constant) ε	BHA Recovery, ppm[a]	
		With Water[b]	Without Water
Petroleum ether	1.89	8.8 (0.7)[c]	Trace
Benzene	2.60	8.1 (0.7)	Trace
Ethyl ether	4.34	7.8 (0.7)	Trace
Acetone	20.70	8.3 (0.7)	Trace
Ethanol	24.30	9.5 (0.7)	Trace

[a]ppm are expressed on a dry weight basis.

[b]Hydration ratio 1:5 granules to water w/w: time 10 min; 30 ml of solvent collected.

[c]Estimated error.

Table 5. Recovery of BHA from Dehydrated Potato Granules on Chromatographic Columns and Büchner (Fritted Glass) Funnels

Solvent	Solvent Volume, ml[b]	BHA Recovery, ppm[a]	
		Columns	Funnels
Petroleum ether	30	8.8 (0.7)[c]	6.9 (0.7)
	60	1.2 (0.8)	2.9 (0.8)
	90	trace	trace
Benzene	30	8.1 (0.7)	8.1 (0.7)
	60	1.9 (0.8)	1.9 (0.8)
	90	trace	trace

[a]ppm are expressed on a dry weight basis.

[b]Potato granules were rehydrated after each 30 ml solvent extraction.

[c]Estimated error.

solvent has proven to be necessary to release the remaining antioxidant. Practically the same results were obtained using pet. ether or benzene and also when granules were treated on a fritted glass funnel instead of a chromatographic column.

Comparative analyses of antioxidants retained in a potato granule process were performed using the short extraction procedure developed in this study. Results for A-B granules are presented in Figure 6. Most importantly, the data did not differ significantly when benzene was substituted by pet. ether as a matter of convenience for the standard GLC assay.

Detection limits in differential pulse voltammetry were 0.2 and 2.0 ppm for BHA and BHT, respectively, while the limits for GLC were 0.2 ppm for both antioxidants. Therefore, the latter technique appeared to be more promising in monitoring a mixture of minute quantities of antioxidants.

D. *Natural Potato Phenolics*

Among the major constituents always present regardless of potato cultivar are L-tyrosine, and chlorogenic and caffeic acids (Matheis and Belitz, 1977). The first two phenolics were found in this study to be extracted in high amounts when water-hot ethanol was the solvent system. Since their anodic wave peak potentials are close to BHA and/or BHT, they would interfere in voltammetric quantitation.

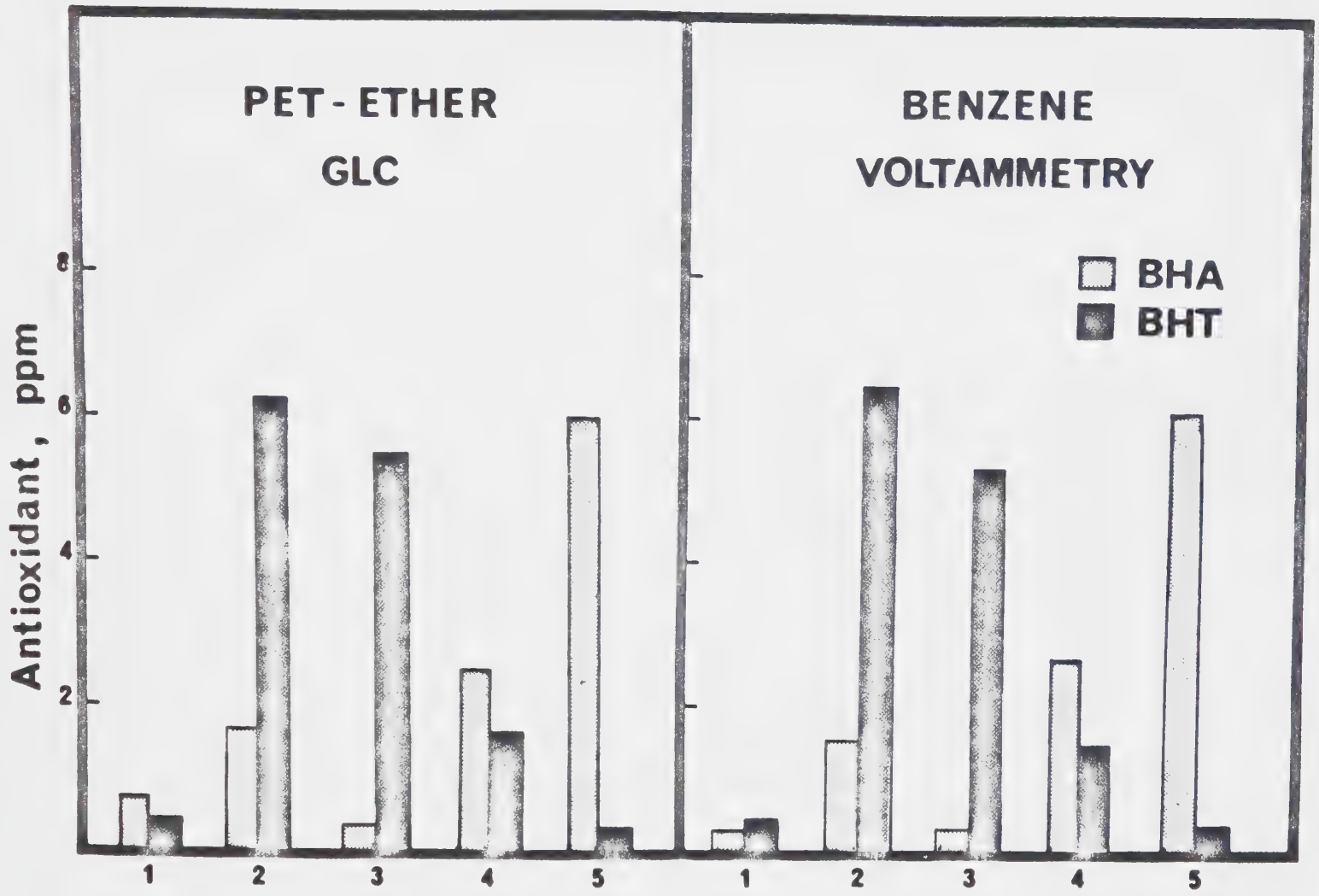


Figure 6. Antioxidant content in freshly made Add-Back granules.

Anodic wave peak potentials of some phenolic compounds present in potatoes are listed in Table 6 . The nonpolar solvent-water phase separation, required in the BHA (BHT) extraction technique developed in this study, was performed in order to remove the natural potato phenolics. Consequently, no interference from natural potato phenolics was encountered in voltammetric or gas-chromatographic assays.

In addition to well-defined wave peak potentials for BHA (BHT) and major potato phenolics (Figure 7), it was found that a plot of peak heights vs concentration gave a linear calibration curve (Figure 8). When the surface of the glassy carbon electrode was not polished between recording of each voltammogram, the slope values of the standard curves decreased very rapidly. Thus, polishing of the electrode surface between each voltammogram was considered as a key step in voltammetric assay. Results obtained from linear regression analyses, and the voltammetric detection limits of the assessed phenolic compounds are recorded in Table 7 .

An extraction technique was developed to attempt to estimate the amount of chlorogenic acid present in dehydrated potato granules. Ethanol proved to be the most effective of the high polarity solvents tested. Nevertheless, the hydration step had to be retained. As seen from Table 8 , weight ratios of 1:2 to 1:4 during cold (room temperature) water hydration showed no significant

Table 6. Anodic Wave Peak Potentials of Some Phenolic Compounds Present in Potatoes

Added as Antioxidant		Naturally Present	
BHA	0.74	Caffeic acid	0.61
BHT	1.05	Chlorogenic acid	0.65
		Ferulic acid	0.84
		α-tocopherol	0.51
		L-tyrosine	1.18

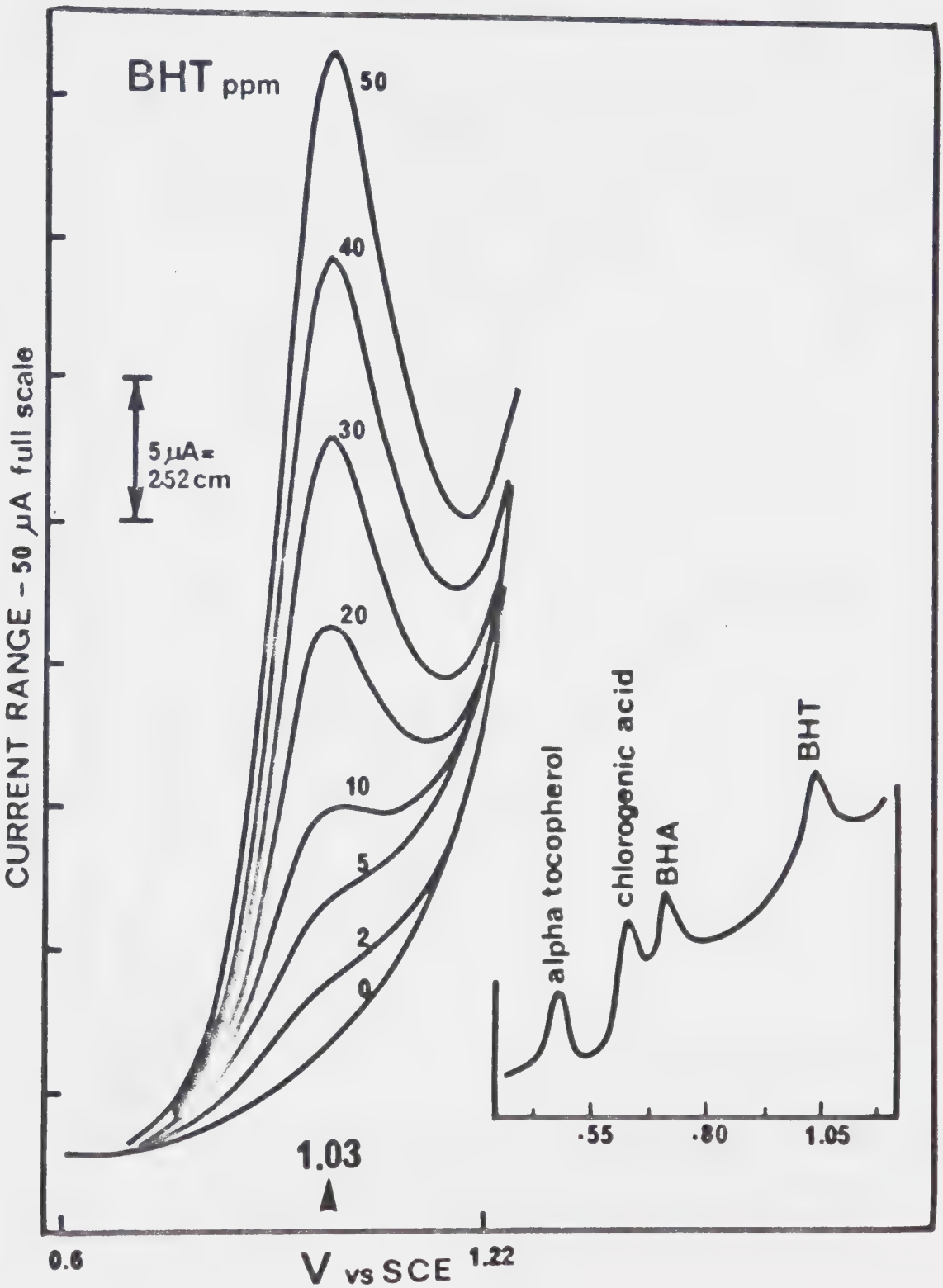


Figure 7. Polarographic recording of various concentrations of BHT, and of other phenolic compounds, showing different wave peak potentials.

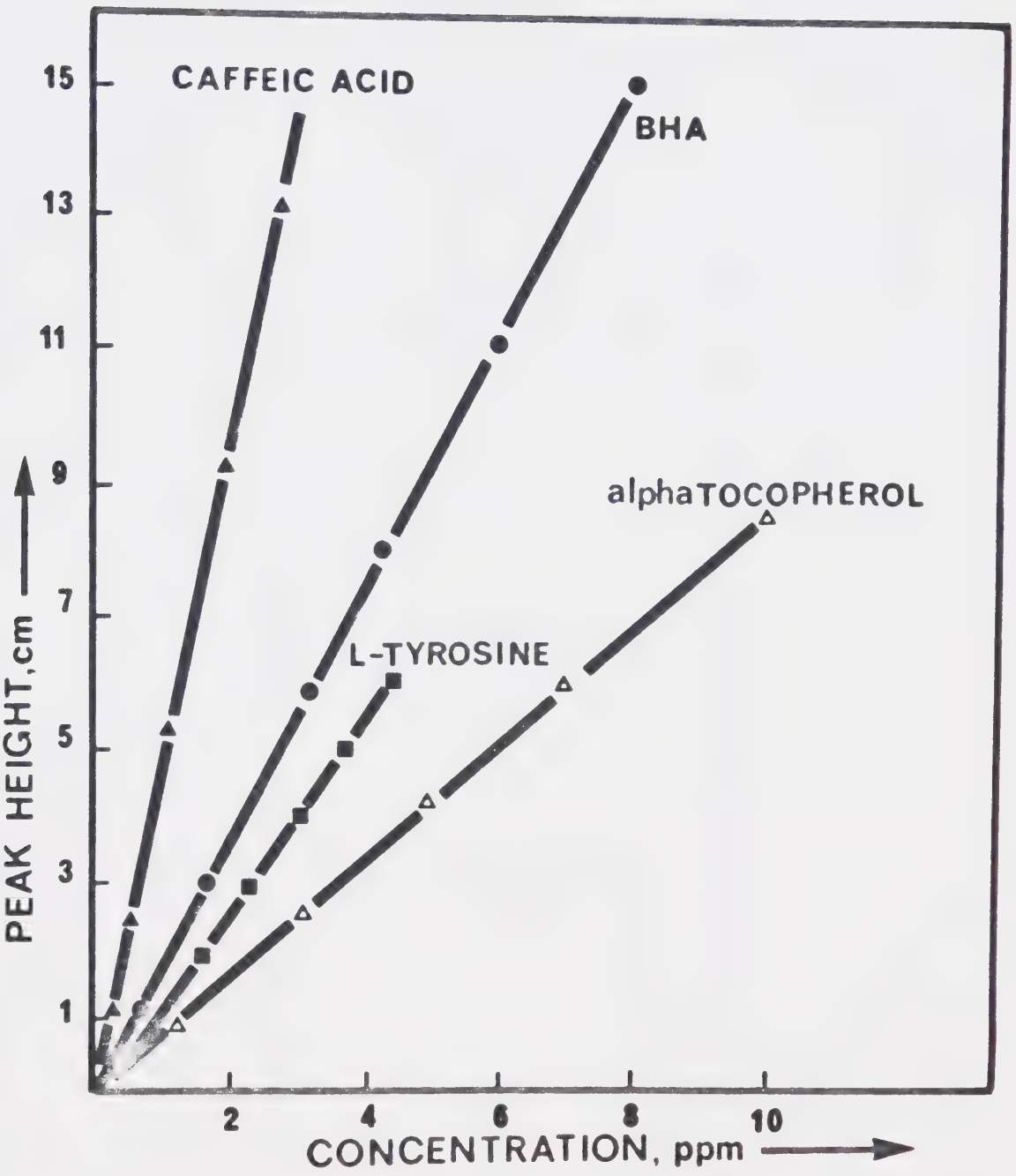


Figure 8. Voltammetric calibration curves for some phenolic compounds.

Table 7. Best Least Squares Fit Coefficients for Voltammograms of Some Phenolic Compounds[a]

Phenolics	Intercept (a)	Slope (b)	Coefficient Correlation (p 0.01)	Detection Limit (ppm)
BHA	-0.1637	1.9210	0.9996	0.2
BHT	-0.4714	0.4230	0.9997	2.0
Caffeic acid	0.0715	4.7603	0.9987	0.1
Chlorogenic acid	-0.1823	1.4397	0.9964	0.2
α-tocopherol	-0.0046	0.8514	0.9997	0.5
L-tyrosine	-0.1600	1.3900	0.9977	0.2

[a] $X = \frac{Y - a}{b}$; where X = phenolic concentration (ppm), Y = peak height (cm).

Table 8. Chlorogenic Acid Content, ppm Dry Weight Basis, obtained from Dehydrated Potato Granules by a Hydration Step followed by Hot Ethanol Extraction

				Hot Water (1:4 w/w)		
	Cold Water			Hydration Time, Min		
Sample	1:2[a]	1:3	1:4	10	15	20
I	51.4	46.7	47.9	68.4	95.9	95.2
II	51.6	54.1	57.8	72.4	96.6	95.8
$\bar{X}$ [b]	51.5	50.4	52.8	70.4	96.2	95.5
SD[c]	±0.1	±5.2	±7.0	±2.8	±0.5	±0.4

[a]Hydration ratio, w/w, granules to water (moisture content, 7.2%). Hydration time was kept constant (10 min) for all cold water hydrations.

[b] $\bar{X}$ = mean.

[c]SD = standard deviation.

difference. The chlorogenic acid actually extracted was only 53.6% of the total content. Unlike cold, hot water (80°C, 1:4 w/w) in 10 min hydration followed by hot ethanol, provided 73.1% extraction; and 15-20 min hydration, 100%.

As emphasized by Bieth et al. (1978), a hydration step is a prerequisite for correct results in a continous cold or hot extraction method for dehydrated granules. They demonstrated that extraction with dry product was always incomplete, and that unreliable results were obtained using pet. ether or carbon disulphide. Their best choice was ethanol, despite the fact that it also extracted low molecular weight natural potato phenolics (Baruah and Swain, 1959; Matheis and Belitz, 1977). At least 15 phenols (free or glucosides) might be extracted.

It appears that two forms of phenolics occur in raw potatoes (Morozova et al.,1974). No attempt was made within the scope of this study to monitor qualitatively and quantitatively all the natural phenolics in raw and dehydrated potatoes. As found in parallel studies, in addition to solvent choice, potato tissue sampling could also be a source of discrepancy in results. Enrichment of the peel and underlying tissue with natural phenolics is a known fact (Reeve et al.,1969; Amberger and Schaller,1973), and even storage temperature of tubers can have an effect (Hasegawa et al.,1966; Mondy et al.,1966)

Nevertheless, voltammetric assay of natural phenolics in dehydrated granules was simple and rapid. It was not as

long as methods using chromatographic separation on paper or columns (Diemair and Huck,1962; Walter and Purcell,1979), and was more specific for chlorogenic acid than the UV-spectrophotometric assay based on bathochromic shift in alkaline solution (Hughes et al.,1962). However, it appears not to be as selective for chlorogenic isomers as a high pressure liquid chromatography method (Walter et al.,1979).

CONCLUSION

Studies on the behaviour of freeze-dried major constituents of potato showed that association of the amylose moiety is responsible for the strong antioxidant entrapment within potato granules. X-ray diffraction data on model systems further indicated that BHA (BHT) recovery from granules is hampered by leached-out amylose chains, rather than by inclusion into an amylose clathrate compound. The conditioning step of the commercial Add-Back process, where the mashed potatoes are held in a stream of cool air for 30-60 min, is believed to cause association of the free amylose and, thereby, the physical entrapment of antioxidants within the realigned molecular network.

The rapid extraction procedure developed in this study essentially releases the immobilized antioxidant upon reconstitution with water. Hydration was proven to be necessary to push the associated amylose chains apart, allowing the solvent access to the antioxidant. The procedure, lasting close to 30 min, involved extraction with organic solvent of the fully rehydrated samples (1:5 w/w, granules to water), followed by removal of water and concentration of the organic phase containing the antioxidant.

Data for BHA content in the granules obtained with and without a rehydration step suggested that the current methods of incorporation of antioxidant in a granule process

are not efficient since antioxidant was shown to be held within the amylose matrix rather than being associated with potato lipids (a major source of rancidity in granules).

GLC and voltammetry were proven to be reliable for quantitation of the extracted antioxidants. Pet. ether was the extraction solvent for GLC assay; addition of a keeper (paraffin oil) to the extract was required prior to evaporating just to dryness. Benzene was chosen for differential pulse voltammetric assay since it was a component of the supporting electrolyte. Peak potentials were +0.74 V for BHA and +1.05 V for BHT. Detection limits in voltammetry using a glassy carbon electrode were 0.2 and 2.0 ppm for BHA and BHT, respectively, while for GLC the limits were 0.2 ppm for both antioxidants. Therefore, the latter technique appeared to be more promising in monitoring a mixture of minute quantities of antioxidants.

Also, the rapid extraction procedure provided means of isolation and quantitation of natural phenolics such as caffeic, chlorogenic and ferulic acids, α-tocopherol and L-tyrosine. As assayed by chlorogenic acid as a marker, a hot instead of cold water rehydratation in a ratio of 1:4 w/w followed by a hot ethanol extraction step provided the most reliable results for natural phenolics of the potato.

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